Antibacterial Bioagents Based on Principles of Bacteriophage Biology: An Overview

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Bacteriophages were discovered almost a century ago. With the advent of antibiotics, the use of bacteriophages for treatment of infections fell out of favor in Western medicine. In light of the rise of antibiotic resistance, phages and their products (lysins) are rediscovered as antibacterial bioagents. This overview summarizes principles of phage biology and their translation for therapeutic and preventive applications. Examples are presented to highlight their therapeutic promise for prophylaxis and treatment of bacterial infections including multi-drug-resistant organisms in humans and animals, and their use as decontaminants of food supplies and environments. Besides research on the in vivo behavior of phages and lysins, dialogues between researchers and regulatory agencies are necessary to publish guidelines for bacteriophage manufacturing and formulation for human use. Only well-designed, double-blind randomized controlled trials will determine if phages and lysins are safe and effective adjuncts or alternatives to antibiotic therapy for infections with multidrug-resistant organisms.

**Keywords.** bacteriophages; lysins; multidrug-resistant bacteria.

The World Health Organization (WHO) has declared antimicrobial resistance as one of the greatest threats to human health [1]. There is urgent need for development of antimicrobial agents; however, few are in the pharmaceutical pipeline, and the number of new antibacterial drugs approved for marketing in the United States continues to drop [2].

Increased understanding of commensal microorganisms suggests that maintaining the microbiome provides a key to preventing colonization and infection with multidrug-resistant (MDR) organisms [3]. Hence, current strategies employed to combat the rise of MDR organisms focus on a more judicious use of antibiotics, and methods to restore indigenous microbiota. In addition, the discovery of microbiome-sparing antimicrobial therapy should be emphasized.

In this context, the interest in bacteriophage therapy has been renewed. This overview serves to outline potential applications of bacteriophages (phages) and their lysins for infection prevention and therapy.

**METHODS**

A literature search was performed using Medline (National Library of Medicine, Bethesda, Maryland; years 1966–2013) and Embase (Elsevier, Amsterdam, The Netherlands; years 1950–2013). Keywords used were bacteriophage, lysin, multidrug resistant bacteria, biofilm, treatment, prevention, prophylaxis. Search was limited to English-language publications. Given the word and reference limitations of this overview article, only a selection of the literature reviewed was included.

**PRINCIPLES OF PHAGE BIOLOGY**

Phages are bacterial viruses and occupy habitats where bacteria thrive. Phages are classified into 13 families according to morphology, type of nucleic acid, and...
presence or absence of an envelope or lipid. The majority of phages are “tailed phages,” composed of an icosahedral head and tail. All tailed phages have double-stranded DNA. According to the morphologic features of the tail, they are classified into 3 families, Myoviridae, Siphoviridae, and Podoviridae [4].

Two phage types can be differentiated according to how they infect their host bacteria (Figure 1). Temperate phages undergo a lysogenic cycle during which they integrate their chromosomes into the bacterial genome and stay in a dormant state as a prophage [4]. When the host bacterium encounters circumstances leading to DNA damage, prophages may be activated and turn on the lytic cycle. Temperate phages harbor the risk of altering the phenotype of their target bacteria [5–8]. They are a key driver in the acquisition of virulence factors in enteric bacteria and of the complex regulating networks that control their expression [5]. Examples are enterohemorrhagic Escherichia coli that contain prophage-encoded Shiga toxins [6], and the Vibrio cholerae prophage-encoded Cholera toxin [9]. Cryptic prophages of E. coli have been shown to encode genes for resistance to antibiotics and other environmental stressors [7, 8]. For these reasons, temperate phages are not considered for therapeutic applications, unless they have been genetically modified [10].

Obligate lytic phages follow an infectious cycle whereby they multiply exponentially in the bacterial cell (Figure 1). During the final stage of multiplication, most tailed phages produce a

**LYTIC PHAGE ISOLATION AND DOSING**

Naturally occurring lytic phages are isolated by enrichment of samples of their natural habitat (ie, sewage or patient material) with the respective bacterial strain to which they are specific [11]. After incubation, lytic plaques are recovered, and undergo purification and further propagation in the bacterial host. Phages are genetically characterized to exclude lysogenic cas- settes, undesired genes, and known virulence factors [11]. The basic technology is well established. Monophage preparations or phage cocktails containing several phages targeting different strains of the same pathogen, and/or pathogens of different bacterial species are used for treatment. Phage cocktails minimize the potential for resistance as phage receptors on the bac-
terial wall are highly phage specific [11]. For this reason, phage cocktails should be preferentially used.

Phages can be applied as local or systemic therapy. Conventional pharmacokinetics do not apply, because phages behave as an exponential self-amplifying drug. Whether or not their amplification is dependent on a distinct threshold in the density of susceptible bacteria, only above which their number increase, is an area of contention. However, the success of phage therapy in acute infection is determined not only by the type of phages and bacteria involved, but also by the actual bacterial density at the time of application, and their proliferation kinetics. The timing of treatment is less crucial in chronic infections where there is an abundance of bacteria [12, 13]. Several mathematical models based on in vitro studies exist to describe the interaction between phage and bacteria [14, 15]; however, in vivo data are needed to validate them. Trials to date have used bacteriophage doses between 10^5 and 10^9 plaque-forming units (PFU) [16–18].

Phage therapy has been used for treatment in Russia, the Republic of Georgia, and Poland since the early 20th century [19, 20]. However, a systematic proof of efficacy, documentation of adverse effects, pharmacokinetics, and dosing in animals and humans was not done, a prerequisite for approval of phages as antibacterial drugs by the US Food and Drug Administration (FDA) and Europeans Medicines Agency (EMA). More recent studies are addressing some of these areas and will be discussed below.

### Table 1. Comparison of Bacteriophages, Endolysins, and Antibiotics as Systemic Antimicrobials

<table>
<thead>
<tr>
<th>Factor</th>
<th>Bacteriophages</th>
<th>Endolysins</th>
<th>Antibiotics</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clinical action</td>
<td>Bactericidal</td>
<td>Bactericidal or bacteriostatic</td>
<td></td>
</tr>
<tr>
<td>Ability to clear biofilm</td>
<td>Yes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Site of action</td>
<td>Concentrates at the site of infection</td>
<td>Do not necessarily concentrate at the site of infection</td>
<td>Do not necessarily concentrate at the site of infection</td>
</tr>
<tr>
<td>Antibacterial spectrum and its consequences</td>
<td>• Target specific</td>
<td>• Target specific</td>
<td>• Broader</td>
</tr>
<tr>
<td></td>
<td>• Cannot be used for empiric therapy</td>
<td>• Cannot be used for empiric therapy</td>
<td>• Can be used for empiric therapy</td>
</tr>
<tr>
<td></td>
<td>• Narrow spectrum can be overcome by</td>
<td>• Only effective against gram-positive</td>
<td>• Spectrum drug specific</td>
</tr>
<tr>
<td></td>
<td>polyvalent phage cocktails</td>
<td>organisms</td>
<td>• Can cause collateral damage to indigenous flora</td>
</tr>
<tr>
<td></td>
<td>• No collateral damage to indigenous flora</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Risk for resistance development</td>
<td>• Inducing factors predominantly known</td>
<td>No resistance known</td>
<td>• Inducing factors predominantly known</td>
</tr>
<tr>
<td></td>
<td>• Monophage preparations: high</td>
<td>at present</td>
<td>• Depending on the biochemical mechanism(s) not limited to targeted bacteria and single antibiotic classes</td>
</tr>
<tr>
<td></td>
<td>• Phage cocktails: lower</td>
<td></td>
<td>• Development of novel antibiotic drugs effective against resistant bacteria time consuming</td>
</tr>
<tr>
<td></td>
<td>• Development of resistant variants can be</td>
<td></td>
<td>• Resistant bacteria might have higher or decreased fitness and/or virulence</td>
</tr>
<tr>
<td></td>
<td>overcome through selection of new phages</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>targeting resistant host variant within days</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>• Resistant mutations might negatively impact bacterial fitness or virulence</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Side effects</td>
<td>Unknown</td>
<td>Unknown</td>
<td>Sufficiently known</td>
</tr>
<tr>
<td>Efficacy</td>
<td>Unproven</td>
<td>Unproven</td>
<td>Proven</td>
</tr>
</tbody>
</table>

### POTENTIAL APPLICATIONS FOR THERAPY AND PREVENTION

#### Efficacy and Safety in Animals

Efficacy and safety studies have been conducted using different animal models, bacteria/phage combinations, and focusing on various infections. A few examples of intraperitoneal and inhaled administered phage therapy in mice will be described below to highlight the importance of dose, timing, and route of administration.

Efficacy studies of single intraperitoneal phage (ENB6) injections for therapy of fatal vancomycin-resistant *Enterococcus faecium* sepsis within 48 hours showed 100% survival of mice at a dose of multiplicity of infection (MOI; the ratio of adsorbed or infecting phages to total bacteria) of 0.3–3.0 if administered 45 minutes after intraperitoneal inoculation, whereas an MOI of 0.003 and 0.03 resulted in survival rates of 40% and 60%, respectively. Treatment with an MOI of 3.0 could be postponed up to 5 hours to rescue 100% of animals, but beyond that point morbidity increased and mortality occurred. However, even with delays of 18 and 24 hours, at which point all the mice were moribund, 50% of the animals went on to recover completely [21].

The ENB6 phage used was immunogenic. Titers of immunoglobulin G (IgG) and immunoglobulin M (IgM) raised 3800-fold and 5-fold, respectively, after the third in a series of
5 monthly intraperitoneal injections at a dose of $10^{10}$ PFU. Thereafter, IgG levels did not change significantly. No anaphylactic or other adverse reactions were observed. Neutralization of phages was not evaluated. Similar results have been obtained using a single intraperitoneal injection of a phage cocktail to rescue mice with fatal *Pseudomonas aeruginosa* infection [22].

Inhalation of phages for difficult to treat lung infection were tested in mouse lung infection models. Intranasal inhalation of phages (MOI 10–100) infected with a lethal *P. aeruginosa* inoculum was curative in a limited time frame after infection (hours 2, 4, and 6: survival of 100%, 75%, and 25%, respectively) [12, 13]. Released bacterial debris was not proinflammatory [13]. If a single high phage dose was administered 24 hours before a lethal intranasal bacterial challenge, 100% of animals were protected, whereas all animals died after an equivalent dose of heat-inactivated phages. The rate of elimination of phages in lungs of uninfected mice was about half-log per day, suggesting that preventive phage treatment might be still efficacious if infection occurs days later. Active and heat-inactivated phages gave rise to identically low but significantly higher levels of inflammatory markers compared to diluent administration, dependent on purity of phage preparation [13].

In an acute nonlethal *Burkholderia cenocepa* pulmonary infection, a single intraperitoneal phage injection 24 hours after inoculation reduced bacterial density and inflammatory marker levels in lungs of mice at day 3 of infection [23]. Intranasally applied phages were ineffective under this condition, corroborating the results of Debarbieux et al [12] and Morello et al [13]. The difference in efficacy of intraperitoneally vs intranasally administered phage models is unexplained. It has been hypothesized by Carmody et al that it might be due to inability of phages to reach bacteria located in the lung interstitium [23].

**Efficacy and Safety in Humans**

The first studies of phage therapy in humans adhering to standards of the FDA and EMA have involved topical and oral phage applications. Interestingly, some phage cocktails gained FDA approval as food decontaminants.

**Topical Phage Therapy**

Chronic otitis caused by *P. aeruginosa*, a difficult-to-treat infection due to biofilm production and drug resistance, has been the target of a prospective, randomized, double-blind phase 1/2a trial of phage therapy in humans, with the clinical outcome as the primary outcome measure and bacterial counts as the secondary outcome measure [16]. A 6-phage cocktail at a dose of $10^5$ of each phage was used. Patients’ isolates were sensitive to at least 1 of those phages. Twenty-four patients, randomized 1:1, were either treated with a single dose of phage cocktail or the diluent used as placebo. Significant clinical improvement from baseline measured with a visual analogue scale ($P = .001$) and significant reductions in *P. aeruginosa* counts ($P < .0001$) were seen compared to placebo. There were no reportable side effects or evidence of local or systemic toxicity. Median duration of phage replication was 21 days, and clearance of all phages was observed after resolution of the infection.

A phase 1 prospective, randomized, double-blind study evaluated the safety of an 8-phage cocktail for treatment of venous leg ulcers [17]. The cocktail contained phages for *P. aeruginosa*, *Staphylococcus aureus*, and *E. coli* at a concentration of $1 \times 10^9$ PFU/mL of each of the component monophages. Forty-two patients with full-thickness venous leg ulcers of >30 days’ duration, with or without clinical signs of infection, were included in the study and randomized 1:1. Either a phage preparation or sterile saline was applied weekly using an ultrasonic debride ment device. No evidence for local or systemic toxicity was observed.

**Phages for Prevention of Foodborne and Waterborne Illnesses**

Phages have been isolated from many food products, and in high numbers from fermented products such as yogurt, cheese, and sauerkraut [24]. In 2006 the FDA approved ListShield, a phage cocktail that can be sprayed on food to reduce *Listeria* contamination [25]. EcoShield, a phage cocktail targeting *E. coli* O157:H7, gained FDA approval in 2011 [25]. FDA clearance for GRAS (Generally Recognized as Safe) is currently pending for a phage cocktail targeting *Salmonella enterica* (SalmoFresh) [25]. Veterinary use of phages for colonization prevention, decolonization, and treatment of infections in animal husbandry is an area of ongoing research [26–28].

Phages appear to play a predominant role in ending cholera epidemics, as the prevalence of environmental *V. cholerae* phages inversely correlates with seasonal cholera epidemics [29]. Research to determine efficacy of phage cocktails for drinking water decontamination in epidemic areas would be of interest. *Vibrio* phage treatment as prevention has shown ameliorated disease in a rabbit model of cholera infection [30].

**Phages for Treatment of Diarrheal Illnesses**

Oral phage therapy is being evaluated as a treatment option for diarrheal illnesses due to contaminated water [18, 31]. Human trials have thus far focused on *E. coli* [18, 31]. Two safety trials, one with a single T4 phage at doses of $3 \times 10^7$ and $3 \times 10^8$ PFU/mL/day × 2 days and one with a T4-like cocktail (9 phages) at a dose of $3 \times 10^8$ PFU/mL/day × 2 days were carried out in healthy volunteers (15 and 30 subjects, respectively) [18, 31]. No adverse effects were reported. Phage-positive stools to an *E. coli* indicator strain increased with dose [18, 31]. Neither T4 phage nor antibodies to the phage were detected in the serum of patients [18, 31]. A trial to evaluate the safety and efficacy of a phage cocktail to treat enterotoxigenic *E. coli*– and
enteropathogenic *E. coli*-induced diarrhea in children in Bangladeshi ongoing (clinicaltrials.gov identifier: NCT00937274). An important consideration is that massive release of lipopolysaccharide from lysed *E. coli* in infected individuals might lead to side effects not observed in safety trials of healthy volunteers.

A relatively recent event exemplifies how phages could be useful for treatment of infections due to problematic enterohemorrhagic *E. coli* strains. In summer 2011, Germany experienced a large epidemic outbreak of foodborne enterohemorrhagic *E. coli* infection. The O104:H4 outbreak caused considerable suffering, 54 deaths, and resulted in a strain on the healthcare and public health systems. Antibiotics were contraindicated, given concern for activation of Shiga toxin expression. Mera-bishvili et al used this *E. coli* strain to demonstrate that rapid selection and genetic characterization of candidate therapeutic phages from environmental sources in the setting of an outbreak is possible within 3 days only [32].

**Effect on Biofilm**

Indwelling medical devices have the propensity to become colonized with biofilm-producing bacteria, which can act as a nidus of infection by evasion of the host immune system and/or tolerance to antimicrobial agents. Phages at individual concentrations (1.0 × 10^10 to 2.2 × 10^10 PFU/mL) were applied prior to, during, after, or repeatedly after overnight inoculation with 10^8 to 10^9 colony-forming units (CFU)/mL *P. aeruginosa* for prevention of in vitro biofilm formation by *P. aeruginosa* on hydrogel-coated Foley catheter segments [33]. If a monophage preparation was used, efficacy was lost within 24 hours due to phage-resistant variants. The use of phage cocktails minimized that risk. Similar studies have shown successful biofilm prevention by other pathogens [34]. Another study demonstrated an interesting and novel mechanism of biofilm prevention via synergism between phage and probiotic bacteria. Pretreatment of a monophage preparation (1 × 10^8 PFU/mL) alone vs pretreatment with *E. coli* HU2117 plus phage to prevent biofilm formation by *P. aeruginosa* on a silicone urinary catheter showed that adherence to catheters was 4 log_{10} units lower when pretreated with *E. coli* and phage at 24 hours and 3 log_{10} units lower at 72 hours in 70% of experiments, and completely prevented in 30% of the experiments compared to no pretreatment [35]. *Escherichia coli* or phage alone had no effect. The lack of efficacy of phage compared to other studies was thought to be due to absence of a hydrogel coating preventing adsorption of phages [35].

In a rat model of orthopedic implant–related methicillin-resistant *S. aureus* (MRSA) and *P. aeruginosa* infection, local bacteriophage injections were combined with intraperitoneal administration of teicoplanin or imipenem/cilastatin plus amikacin [36]. In the MRSA group, the number of CFU units per animal was reduced 10-fold by phage plus antibiotic compared to control. Phage or antibiotic treatment alone was less effective. Biofilm was completely resolved in the MRSA phage plus antibiotic treatment group as measured by electron microscopy indicating synergistic activity. In the *P. aeruginosa* group, biofilm thickness did not differ between treatment groups [36].

**PHAGE ENDOLYSINS**

Lysins are obtained by cloning the lysin cassette of the respective gram-positive bacteria phage species, expression, and purification of the protein. Animal models have shown high specificity and efficacy of native or recombinant lysins for treatment of gram-positive bacterial colonization/infection on mucous membranes without affecting the normal flora [37]. ClyS, a chimeric lysin active against *S. aureus* including MRSA, eradicated methicillin-sensitive *S. aureus*/MRSA significantly better than mupirocin in a skin colonization/infection mouse model [38]. Due to the short half-life, several systemic doses, or constant dosing, or direct application to deep compartments was necessary [37]. Synergy of the lysin ClyS with oxacillin at doses that were not individually protective against MRSA infection have been observed in vivo, indicating further investigation of their use for combination therapies [37].

Lysins are immunogenic; however, antibodies against lysins specific to *Bacillus anthracis* (PlyG), *Streptococcus pyogenes* (PlyC), or *Streptococcus pneumoniae* (Pal) did not neutralize their hydrolytic activity in vitro, nor did antibodies to pneumococcal lysin (Cpl-1) in vivo [39]. Interestingly, resistance to lysins has not been reported to date [37, 39], possibly due to co-evolution of the lytic enzyme binding domain and host bacteria over millennia to target a unique and essential molecule in the bacterial cell wall to enhance phage survival [37]. Cholin, an indispensable cell wall molecule for *S. pneumoniae* and target for pneumococcal lysin, is an example [37].

PlyC, a lysin against *Streptococcus equi*, the causative organism of equine strangles disease, has been evaluated as an environmental disinfectant [39]. It was 1000 times more active on a per-weight basis than commercially available oxidizing disinfectants, and retained effectiveness independent of surface material, presence of nonionic detergents, hard water, and organic material [39]. Lysins active against MRSA and vancomycin-resistant enterococci have been characterized [37–40], and the thought of their use for targeted surface disinfection in hospitals is enticing.

**CONCLUSIONS**

Antibacterial therapy based on phage biology has the potential for many applications, most importantly for the prevention and treatment of infections due to MDR organisms. The work
by Merabishvili et al [32] also argues for the consideration of phage therapy as a potential armament in outbreak situations, and could provide an incentive for competent authorities such as WHO and/or the European and US Centers for Disease Control to create regulatory guidelines and phage banks, to enable physicians to use phages as additional tools in the outbreak treatment of otherwise virtually untreatable infections. The vast experience in use of phages for treatment in Russia signals effectiveness, albeit based on semianecdotal reports. To establish if phages and lysins can be used as adjuncts and/or alternatives to antibiotics, coordinated and funded research programs to study their behavior in vivo under healthy and diseased conditions are needed. Due to space limitation of this overview, areas that need further research and clarification prior to clinical testing are summarized in Table 2. Regulatory agencies should be encouraged by basic and clinical researchers to publish guidelines for manufacturing and formulation of phage cocktails and lysin preparations for human use, as well as guidelines for clinical trials. Only results from well-designed, randomized, blinded and controlled trials will ultimately prove or disprove their efficacy.

Notes

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References


